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Two new stygobiotic species of *Horatia* Bourguignat, 1887 (Hydrobiidae) from Croatia

Andrzej Falniowski[1], Jozef Grego[2], Aleksandra Rysiewska[1], Artur Osikowski[3], Sebastian Hofman[4]

1 *Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland* **2** *Horná Mičiná 219, SK-97401, Banská Bystrica, Slovakia* **3** *Department of Animal Reproduction, Anatomy and Genomics, University of Agriculture in Krakow, Mickiewicza 24/28, 30-059, Kraków, Poland* **4** *Department of Comparative Anatomy, Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland*

Corresponding author: Artur Osikowski (a.osikowski@urk.edu.pl)

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Abstract

In this paper we describe two new species of the freshwater snails of genus *Horatia*. A new stygobiotic species of *Horatia* Bourguignat, 1887 is described from Izvor Beguša in Croatia. It occurs in sympatry with the crenobiotic *H. klecakiana* Bourguignat, 1887, but is morphologically and molecularly distinct. It is characterized by the terminal part of the body whorl separated from the columella, and neither eyes nor any pigment on the soft parts. It is a stygobiont gastropod, known so far only from one living specimen and several empty shells, thus its soft part morphology and anatomy remain unknown. Another new species of stygobiotic *Horatia* was found inside the cave Mali Rumin, its description is based solely on numerous empty shells from the cave sediments.

Keywords

Cytochrome oxidase, open coil, phylogeny, stygobiont gastropod, sympatry

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Introduction

Bourguignat (1887) described the genus *Horatia*, with its type species *H. klecakiana* Bourguignat, 1887, from "sorgente près de Ribaric, dans la vallée de Cetina" in Croatia (Fig. 1A). Radoman (1983) identified this type locality with the Vrijovac spring in the source area of the Cetina River. The Bourguignat's syntype is figured at the Fig. 1A, together with seven nominal species from upper Cetina Basin described by him in 1887. All these species (Fig. 1) were later synonymized by Brusina (1907) with *H. klecakiana* (Radoman 1965, 1966, 1973, 1983), as Bourguignat obviously underestimated the high variability of the species (Glöer and Reuselaars 2020). *Horatia* was the first nominal genus described for the European Hydrobiidae with valvatiform shell (Bodon et al. 2001). Taylor (1966) established Horatiini as a tribe in Hydrobiidae, within the subfamily Cochliopinae, and Bole (1993) established a distinct family Horatiidae. Radoman (1973) included *Horatia* in Orientalinidae. Kabat and Hershler (1993) presented a review of understanding of this genus in the literature. Szarowska and Falniowski (2014) revised the phylogenetic position of *Horatia* applying molecular data, as a sister clade of *Sadleriana* Clessin, 1890. Glöer and Reuselaars (2020) questioned the identification of *Horatia* in Szarowska and Falniowski (2014), as having "closed umbilicus". However, the umbilicus presented in the photographs in Szarowska and Falniowski (2014) is exactly identical with the one presented by Glöer and Reuselaars (2020) for their new species *H. podvisensis* Glöer et Reuselaars, 2020, and with the one of a syntype of *H. klecakiana* (Fig. 1A). Radoman (1983) listed three species of *Horatia* from the former Yugoslavia: *H. klecakiana*, *H. novoselensis* Radoman, 1966, and *H. macedonica* (Kuščer, 1937) (Fig. 2). Species of *Horatia* were reviewed by Schütt (1961), Boeters (1974, 1998) and Bole (1993). The stygobiotic *Horatia knorri* Schütt, 1961 from Spring Ombla near Dubrovnik was later synonymised by the same author (Schütt 2000) with *Pseudamnicola troglobia* Bole, 1961. However, the morphology of the shell of *H. knorri* suggests its species distinctness from *P. troglobia*, as well as its uncertain generic assignment, only provisionally within the genus *Horatia* (Hirschfelder 2017). All the gastropods from Caucasus previously assigned to the genus *Horatia* were transferred to the new genera *Pontohoratia* Vinarski, Palatov & Glöer, 2014 and *Motsametia* Vinarski, Palatov & Glöer, 2014. The hitherto known species of *Horatia* occur in springs (thus being crenobiotic) at western Balkan Peninsula (Fig. 2), having eyes and pigmented mantle, but no species has been hitherto confirmed from stygobiont habitats.

In 2020 we collected one live specimen (Figs 3, 4A) and several empty shells of *Horatia* in Izvor Ruda Beguša, cave just above the spring zone and in the spring lake, sieved from sandy sediment at the spring and cave bottom, 13 km ESE of Sinj, Split District, Croatia. It occurred in sympatry with a few living specimens of *H. klecakiana*. The specimen was evidently a stygobiont, with neither eyes nor pigment, and with the characteristic shell, evidently different from the ones found in the other species of *Horatia*. Another stygobiotic species, represented by numerous empty shells and shell fragments was found inside the cave Mali Rumin, Split District, Croatia (in summer 2020), and earlier in the active spring sediments in the same locality (in spring 2017).

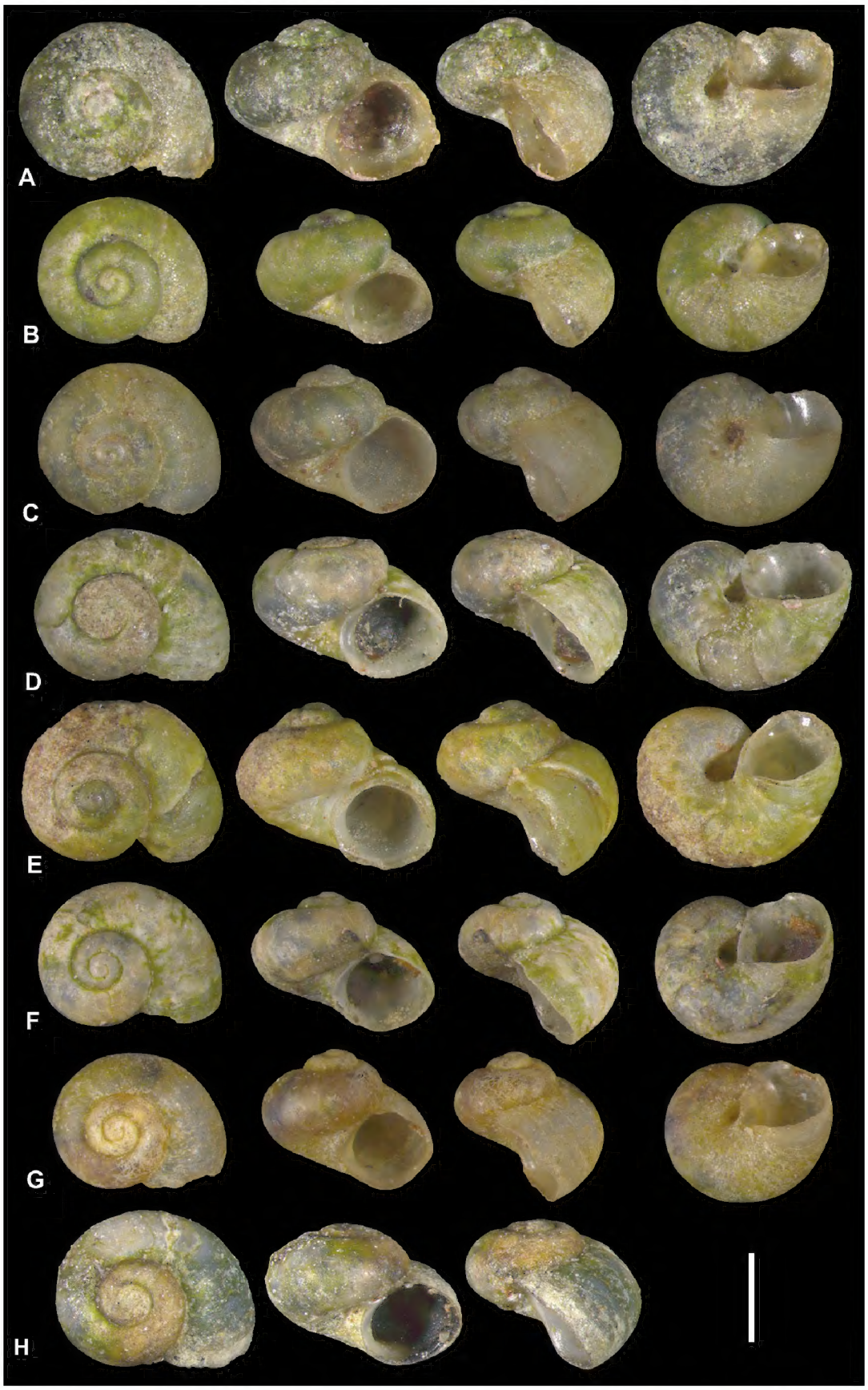

Figure 1. Type material of taxa described by Bourguignat, 1887 **A** syntype of *H. klecakiana* MHNG -110592. Croatie, ex Yougoslavie, Ribarić, Vallée de La Cettina (=Cetina) (=Vrijovac spring in the source area of the Cetina River), Vriovac spring in Paško polje, near to village Cetina. The following species are junior synonyms of *H. klecakiana*: **B** syntype of *H. verlikana* MHNG 110600 Marais entre Verlika (=Vrlika=Verlicca) et Ribaric **C** syntype of *H. palustris* MHNG 110597 Fontaine à Ervac (=Hrvace) **D** syntype of *H. obtusa* MHNG 110596 Sorgente de La Cetina **E** syntype of *H. letourneuxi* MHNG 110593 Fontaine du moulin à Ervac (=Hrvace) **F** syntype of *H. fontinalis* MHNG 110589, Sorgente de La Cettina **G** syntype of *H. albanica* MHNG 110587, Source du moulin Cetina, à Durazzo (=Durrës), not Durrës in Albania, likely the misspelling of the watermill name in village Cetina **H** syntype of *H. obliquq* MHNG 110594 Fontaine du moulin à Ervac (=Hrvace). (Photo MHNG by Estée Bochud and Eike Neubert, kindly provided by Emmanuel Tardy).

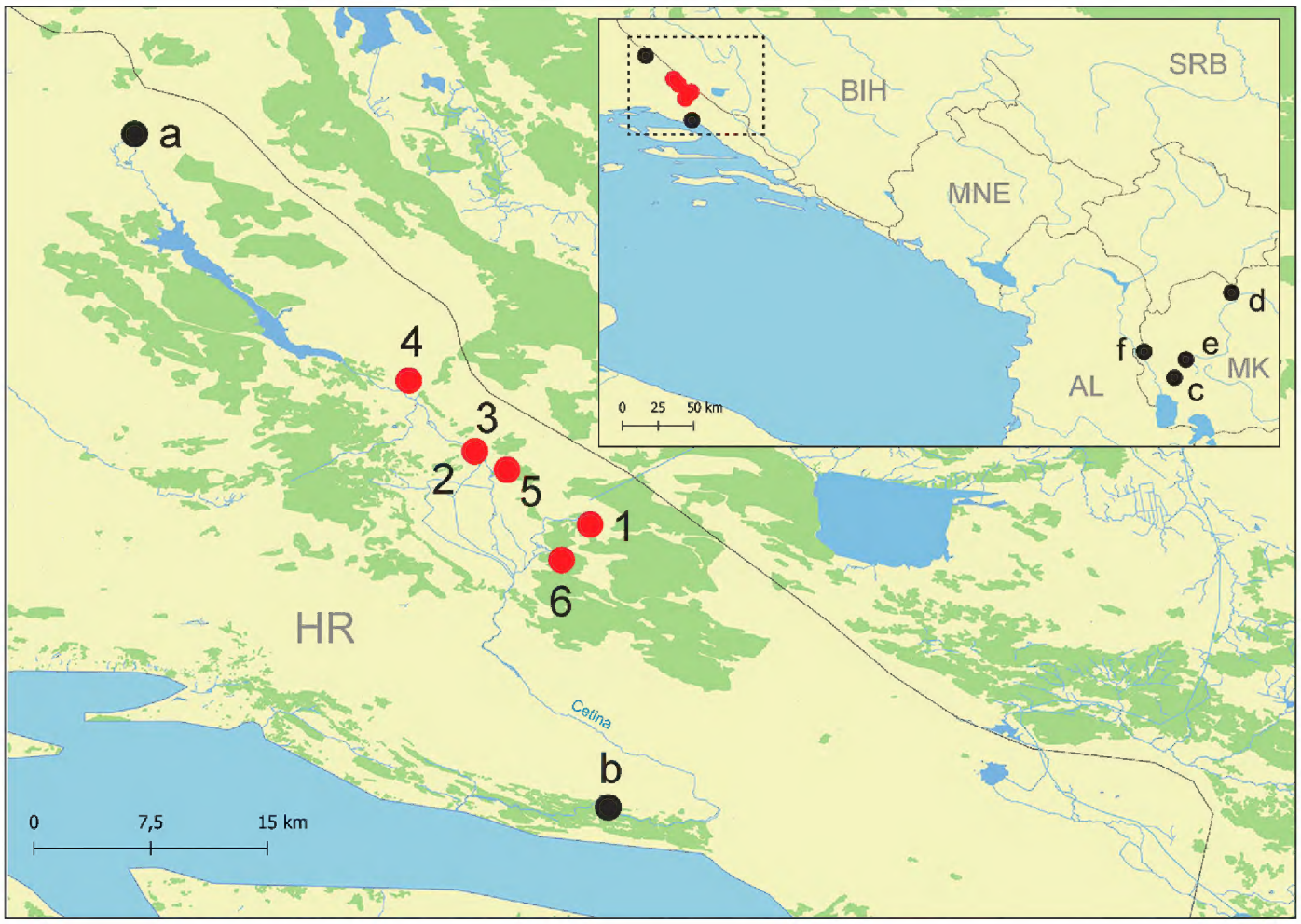

Figure 2. Localities of *Horatia*. Red circles and numbers indicate new *Horatia* localities given in the material description. Black circles and letters: **a** *H. klecakiana* – locus typicus **b** *H. klecakiana* – Kučiće (Szarowska and Falniowski 2014) **c** *H. novoselensis* – locus typicus **d** *H. macedonica* – locus typicus **e** *H. podvisensis* – locus typicus **f** *H. podvisensis* – the other locality (Glöer and Reuselaars 2020). For description of localities 1–6 - see Materials and methods

Material and methods

The live *Horatia* snails and empty shells were collected by A. Falniowski, J. Grego M. Olšavský and J. Olšavská at August 5-th 2020 and by J. Grego, G. Jakab and B. Šmída during March 17-th 2017 at following localities (Figs 2, 3):

1. Izvor Ruda – Beguša, Ruda, sand at the stream bottom below the spring lake, 13 km ESE of Sinj, Split District, Croatia, 43°40'06.6"N, 16°47'45.6"E – *H. ozimeci* sp. nov. and *H. klecakiana* (Fig. 3E).
2. Vrelo Kosinac 1, Sinjski Obrovac, Sinj District, Croatia, 43°43'40.89"N, 16°42'2.29"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3A).
3. Vrelo Kosinac 2, Sinjski Obrovac, Sinj District, Croatia, 43°43'43.11"N, 16°42'0.83"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3B).
4. Mali Rumin cave, Rumin, Sinj District, Croatia, 43°46'50.88"N, 16°38'56.75"E – *H. stygorumina* sp. nov. in cave sandy sediment and *H. klecakiana* at sediment of the cave entrance (Fig. 3C).
5. Gala spring, opposite to Crkva Svih Svetih, Gala, Sinj District, Croatia, 43°42'43.00"N, 16°43'39.88"E – *H. klecakiana* (Fig. 3D).

Figure 3. Sampled localities in Sinj District, Croatia **A** Sinjski Obrovac, Vrelo Kosinac 1 **B** Sinjski Obrovac, Vrelo Kosinac 2 **C** Rumin, cave Mali Rumin **D** Gala, Vrelo Gala **E** Ruda, Izvor Ruda Beguša **F** Grab, Grabske Mlinice, Izvor Grab.

6. Grabske Mlinice, Grab, Sinj District, Croatia, 43°38'27.26"N, 16°46'13.57"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3F).

The snails were collected from the sediment with a 500 µm sieve and fixed in 80% analytically pure ethanol, replaced two times, and kept in -20 °C temperature in a refrigerator. The shells were photographed with a Canon EOS 50D digital camera, under

Table 1. Taxa used for phylogenetic analyses with their GenBank accession numbers and references.

Species	COI GB numbers	References
Agrafia wiktori Szarowska & Falniowski, 2011	JF906762	Szarowska and Falniowski 2011
Alzoniella finalina Giusti & Bodon, 1984	AF367650	Wilke et al. 2001
Anagastina zetavalis (Radoman, 1973)	EF070616	Szarowska 2006
Avenionia brevis berenguieri (Draparnaud, 1805)	AF367638	Wilke et al. 2001
Belgrandiella kuesteri (Boeters, 1970)	MG551325	Osikowski et al. 2018
Belgrandia thermalis (Linnaeus, 1767)	AF367648	Wilke et al. 2001
Dalmatinella fluviatilis Radoman, 1973	KC344541	Falniowski and Szarowska 2013
Daphniola louisi Falniowski & Szarowska, 2000	KM887915	Szarowska et al. 2014a
Ecrobia ventrosa (Montagu, 1803)	KX355839	Osikowski et al. 2016
Fissuria boui Boeters, 1981	AF367654	Wilke et al. 2001
Graziana alpestris (Frauenfeld, 1863)	AF367641	Wilke et al. 2001
Graecoarganiella parnassiana Falniowski & Szarowska, 2011	JN202352	Falniowski and Szarowska 2011
Grossuana angeltsekovi Glöer & Georgiev, 2009	KU201090	Falniowski et al. 2016
Hauffenia tellinii (Pollonera, 1898)	KY087861	Rysiewska et al. 2017
Horatia klecakiana Bourguignat 1887	KJ159128	Szarowska and Falniowski 2014
Iglica gracilis (Clessin, 1882)	MH720985	Hofman et al. 2018
Islamia zermanica (Radoman, 1973)	KU662362	Beran et al. 2016
Montenegrospeum bogici (Pešić & Glöer, 2012)	KM875510	Falniowski et al. 2014
Paladilhiopsis grobbeni Kuscer, 1928	MH720991	Hofman et al. 2018
Pseudorientalia sp.	KJ920490	Szarowska et al. 2014b
Radomaniola curta (Küster, 1853)	KC011814	Falniowski et al. 2012
Sarajana apfelbecki (Brancsik, 1888)	MN031432	Hofman et al. 2019
Tanousia zrmanjae (Brusina, 1866)	KU041812	Beran et al. 2015

a Nikon SMZ18 microscope with dark field; measurements of the shell were taken using IMAGEJ image analysis software (Rueden et al. 2017).

DNA was extracted from whole specimens; tissues were hydrated in tris-EDTA (TE) buffer (3 × 10 min); then total genomic DNA was extracted with the Sherlock extraction kit (A&A Biotechnology), and the final product was dissolved in 20 µl of TE buffer. The extracted DNA was stored at -80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland).

Mitochondrial cytochrome oxidase subunit I (COI) locus was sequenced. Details of PCR conditions, primers used and sequencing were given in Szarowska et al. (2016). Sequences were initially aligned in the MUSCLE (Edgar 2004) Programme implemented in MEGA 7 (Kumar et al. 2016) and then checked in BIOEDIT 7.1.3.0 (Hall 1999). Uncorrected p-distances were calculated in MEGA 7. The estimation of the proportion of invariant sites and the saturation test for entire data sets (Xia 2000; Xia et al. 2003) were performed using DAMBE (Xia 2013). In the phylogenetic analysis, additional sequences from GenBank were used as reference (Table 1). The data were analysed using approaches based on Bayesian Inference (BI) and Maximum Likelihood (ML). In the BI analysis, the GTR + I + Γ model of nucleotide substitution was applied. Model was selected using MRMODELTEST 2.3 (Nylander 2004). The analyses were run using MRBAYES v. 3.2.3 (Ronquist et al. 2012) with default of most priors. Two simultaneous analyses were performed, each with 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of the trees were discarded as burn-in. The

analyses were summarised as a 50% majority-rule tree. Convergence was checked in TRACER v. 1.5 (Rambaut and Drummond 2009). The Maximum Likelihood analysis was conducted in RAxML v. 8.2.12 (Stamatakis 2014) using the 'RAxML-HPC v.8 on XSEDE (8.2.12)' tool via the CIPRES Science Gateway (Miller et al. 2010). We applied the GTR model which is the only nucleotide substitution model implemented in RAxML, whose parameters were estimated by RAxML (Stamatakis 2014).

For comparison purposes the pictures of type material of taxa described by Bourguignat, 1887, were used. The pictures were kindly provided by Emmanuel Tardy (MHNG).

Abbreviations

JG Jozef Grego collection;
MHNG Muséum d'histoire naturellem ville Genéve;
NHMW Naturhistorisches Museum Wien, Austria;
OZRM Croatian Natural History Museum – Opća zbirka recentnih mekušaca, Zagreb, Croatia;
SBMNH Santa Barbara Museum of Natural History, California, USA.

Systematic part

Family Hydrobiidae Stimpson, 1865
Subfamily Horatiinae D. W. Taylor, 1966
Genus *Horatia* Bourguignat, 1887

***Horatia ozimeci* Grego & Falniowski, sp. nov.**
http://zoobank.org/77EA36ED-B25F-4E95-9DC5-20C8B3513B40
Figs 4A–E, 5A

Type locality. Spring Izvor Ruda – Beguša, Ruda, sand at the stream bottom below the spring lake, 13 km ESE of Sinj, Split district, Croatia; 43°40'06.6"N, 16°47'45.6"E.

Holotype. Dry shell with operculum (Fig. 4B), J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11600.

Paratypes. The single live collected paratype (Fig. 4A) has been destroyed for DNA extraction; GenBank number: MW448545.

From type locality: J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg August 5-th 2020, OZRM 11601/1 specimen; NHMW 113607/1 specimen, JG 1542/28 specimens (Fig. 4C–E); Type locality, J. Grego, G. Jakab and B. Šmída leg. March 17-th 2017, JG F0724/24 specimens.

Diagnosis. Shell minute, valvatoid, distinguished from the other species of *Horatia* by its body whorl separated at its terminal sector from the penultimate one (scalarity at this part), the circular and complete peristome and extremely wide umbilicus showing earlier whorls inside.

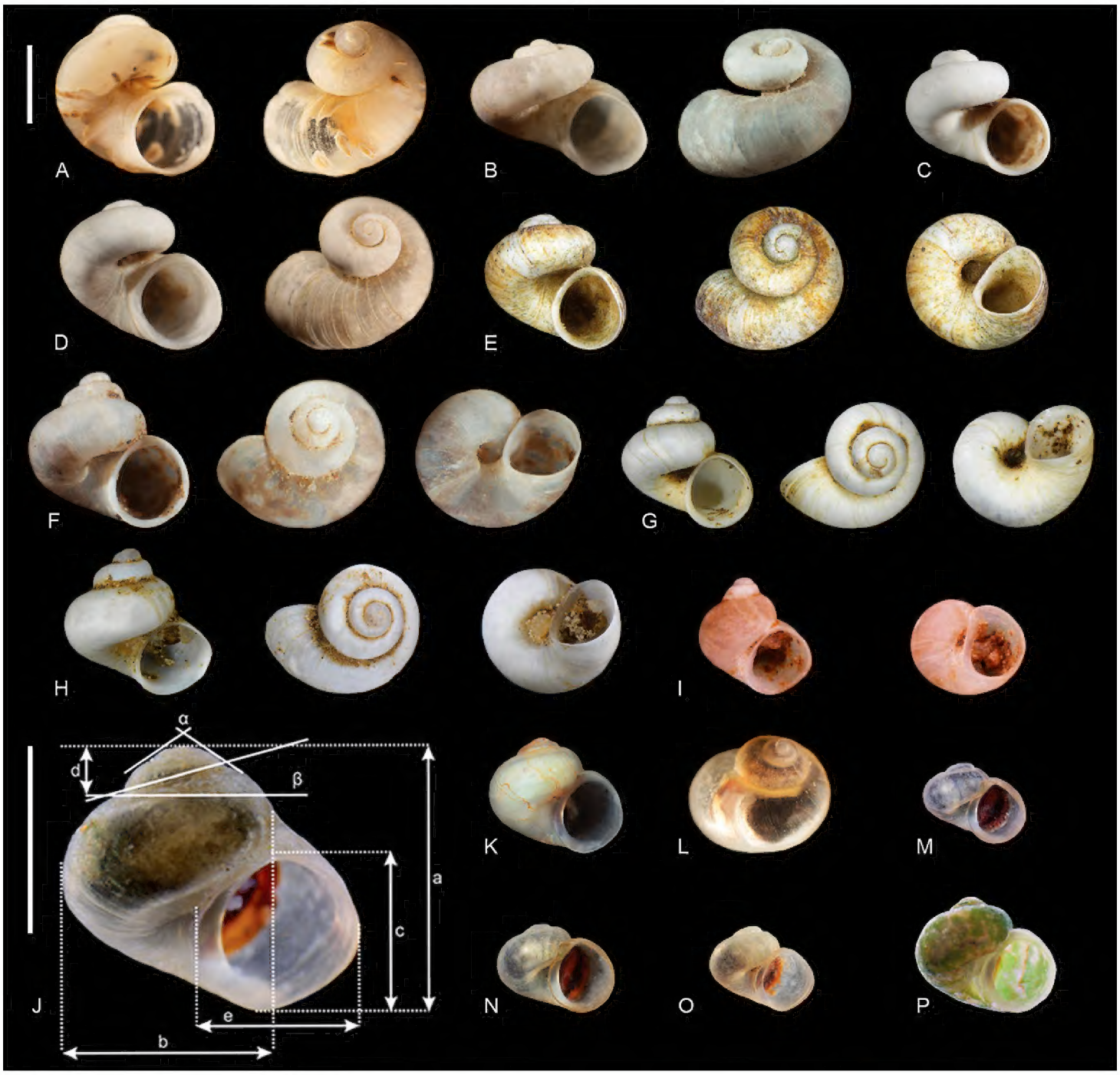

Figure 4. Shell variability of two *Horatia* species **A–E** *H. ozimeci* **A** sequenced specimen (2H76) **B** holotype OZRM 11603 **C–E** paratypes **E** NHMW 113607, (Photo NHMW by Ivo Gallmetzer) **F–H** *H. stygorumina* **F** holotype OZRM 11600 **G–H** paratypes NHMW 113608, (Photo NHMW by Gallmetzer) **I** Cf. *Horatia knorri* Schütt, 1961, spring of Ombla in Komolac, holotype SMF 164247 (Photo SMF by Sigrid Hof) **J–P** sequenced specimens of *H. klecakiana* **J** Vrelo Kosinac 2 **K** *H. klecakiana*, Gala spring **L** *H. klecakiana*, Ruda Beguša **M, N, P** *H. klecakiana*, Vrelo Kosinac 1 **O** *H. klecakiana*, Grabske Mlinice. The shell measurements are also shown: a shell height, b body whorl breadth, c aperture height, d spire height, e aperture breadth, α apex angle, β angle between body whorl suture and horizontal surface. Scale bars: 1 mm.

Description. ***Shell*** (Fig. 4B) 2.14 mm high and 1.62 mm broad, valvatoid, whitish, translucent, rather thin-walled, consisted of about 3 ½ whorls, growing rapidly and separated by moderately deep suture, more prominent closer to the aperture: the terminal part of the body whorl completely separated from the penultimate one. Spire low and narrow, body whorl large. Aperture prosocline, circular in shape, peristome complete and separated from the columella, swollen, umbilicus extremely wide, with

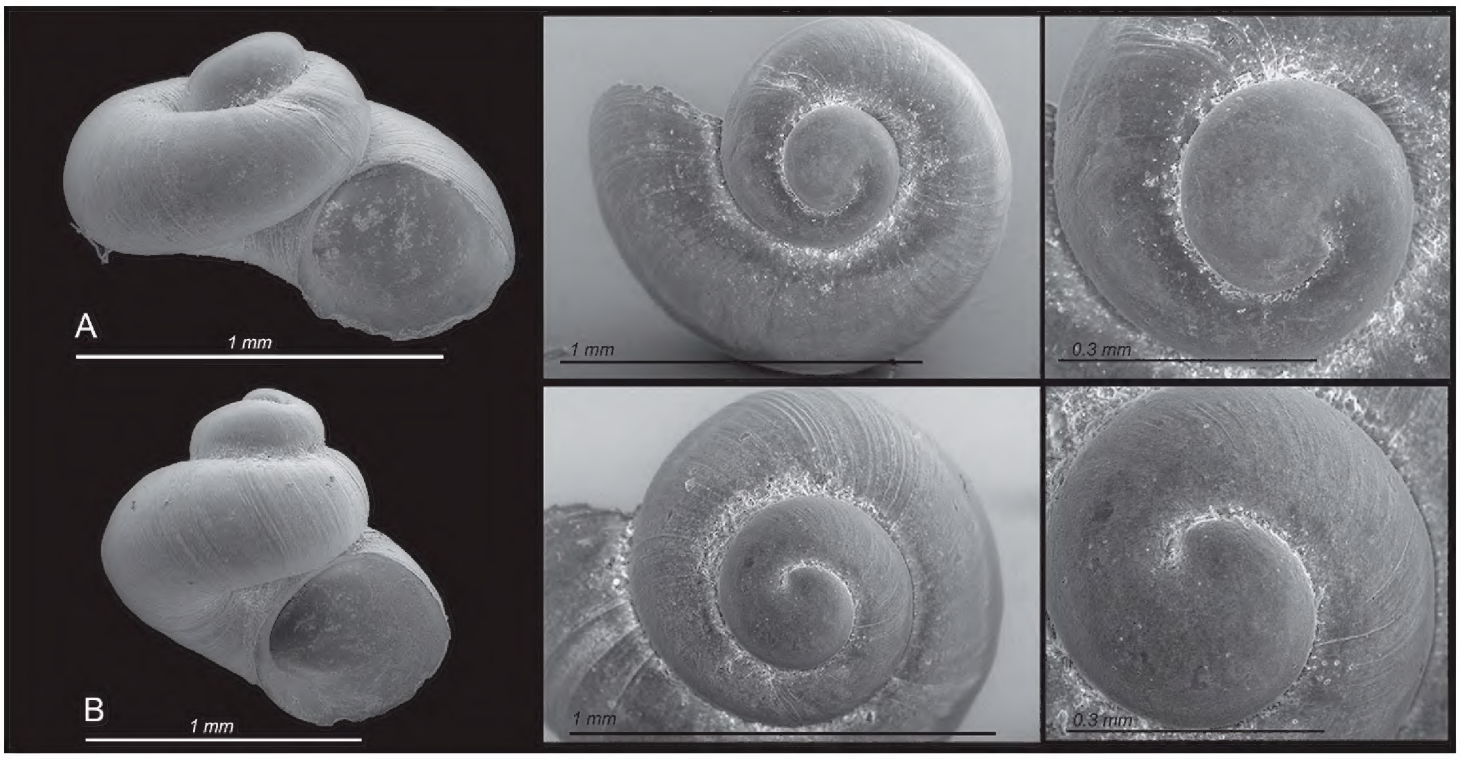

Figure 5. SEM images of *H. ozimeci* (**A**) juvenile specimen from the type locality (SBMNH 635080) and *H. stygorumina* (**B**) juvenile shell from cave Mali Rumin (SBMNH 635079). SEM SBMNH by Vanessa Delnavaz.

the earlier whorls visible inside. Shell surface smooth, with growth lines delicate but visible. Operculum reddish-brown, translucent, paucispiral.

Shell variability restricted (Fig. 4A, C–E), and shell less variable than in *H. klecakiana* (Figs 1, 4J–P).

Protoconch (Fig. 5A) smooth, similar as in *H. klecakiana*.

Measurements of holotype, illustrated paratypes, and shells of *H. klecakiana*: Table 2.

Soft parts morphology and anatomy. The body white, pigmentless, with no eyes. The anatomy unknown.

Derivatio nominis. The specific epithet *ozimeci* refers to our friend Mr.sc. Roman Ozimec, a biospeleologist from Bast, Croatia, deeply devoted to the study and protection of the subterranean habitats in the Dinarides and Balkans.

Distribution and habitat. Known from the type locality. The type locality is a karst spring lake surrounded by three outflow caves intermittently draining the karst conduit at high water saturation. The spring draining water from sinkholes at Buško Jezero (Bosna and Hercegovina) and supports the river Ruda, a left tributary of Cetina River. The following Hydrobiidae species were detected in the habitat: *Horatia klecakiana*, *Orientalina curta germari* (Frauenfeld, 1863), *Montenegrospeum sketi* Grego & Glöer, 2018, *Kerkia jadertina sinjana* (Kuščer, 1933).

Molecular distinctness and relationships of *Horatia ozimeci*. We obtained eight new sequences of COI (409 bp, GenBank Accession Numbers MW448545–MW448552). The tests by Xia et al. (2003) revealed no saturation. The topologies of the resulting phylograms were identical in both the ML and BI. All the seven sequences of *H. klecakiana*, collected at five localities, were identical. *H. ozimeci* formed a sister clade with *H. klecakiana* (bootstrap support 100%, Bayesian probability 1.0), confirming the

Table 2. Shell measurements of the *Horatia*: I–IV *H. ozimeci* (I sequenced specimen, II holotype, in bold; III–IV paratypes); V–VII sequenced specimens of *H. klecakiana*; VIII *H. stygorumina*, holotype, in bold. Measurements are shown in Fig. 4J.

	I	**II**	III	IV	*H. ozimeci*	V	VI	VII	**VIII**
a	2.14	**1.70**	2.02	1.71	1.89 ± 0.22	1.61	1.51	1.61	**2.08**
b	1.62	**1.58**	1.56	1.38	1.54 ± 0.11	1.33	1.19	1.26	**1.57**
c	1.22	**1.12**	1.29	1.05	1.17 ± 0.11	1.14	0.88	1.08	**1.24**
d	0.23	**0.32**	0.18	0.26	0.25 ± 0.06	0.17	0.26	0.25	**0.49**
e	1.05	**1.05**	1.18	0.95	1.06 ± 0.09	0.97	0.91	0.92	**1.08**
α	131	**131**	123	124	127.25 ± 4.35	112	114	113	**104**
β	4	**9**	8	9	7.50 ± 2.38	6	11	10	**8**

congenerity of the two taxa (Fig. 6). Within genus *Horatia* the p-distance between the taxa was 0.074. This well supported clade belongs to the Horatiinae, subfamily of Hydrobiidae. Deeper relationships within the Horatiinae remain unresolved, because of the lack of acceptable support for deeper nodes, which is typical of the phylograms based on COI.

Horatia stygorumina **Grego & Rysiewska, sp. nov.**

http://zoobank.org/36A97AFE-52B6-4F52-A17E-348C5CF604F3

Figure 4F–H, 5B

Type locality. Mali Rumin cave, Rumin, Sinj District, Croatia, 43°46'50.88"N, 16°38'56.75"E.

Holotype. Dry specimen, J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11602 (Fig. 4F).

Paratypes. From Type locality: J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11603/1 specimen; NHMW 113608/1 specimen, JG F1526/160 specimen; Type locality, J. Grego, G. Jakab and B. Šmída leg. March 17-th 2017, JG F0736/26 specimens.

Other material. Morphologically similar stygobiotic *Horatia* shells were found in following spring localities (springs at left tributaries of Upper Cetina River: Vrelo Kosinac 1, Sinjski Obrovac, Sinj district, Croatia, 43°43'40.89"N, 16°42'2.29"E JG/8; Vrelo Kosinac 2, Sinjski Obrovac, Sinj District, Croatia, 43°43'43.11"N, 16°42'0.83"E JG/4; Grabske Mlinice, Grab, Sinj District, Croatia, 43°38'27.26"N, 16°46'13.57"E JG/3). For the time being we treat those as *H.* cf. *stygorumina*.

Diagnosis. Shell minute, trochiform, distinguished from the other species of *Horatia* by its very wide umbilicus showing earlier whorls inside. From the *H. ozimeci* s. nov. distinguished by higher conical spire and suture reaching the aperture, as well by more swollen protoconch and by slight sinuation at labral columellar margin. From stygobiotic cf. *Horatia knorri* different by much wider umbilicus and by aperture shape less declined from the columella in its labral profile.

Description. ***Shell*** (Fig. 4F) 2.08 mm high and 1.57 mm broad, conical, fresh shells milky white and translucent, with 3 rounded inflated whorls and deep suture,

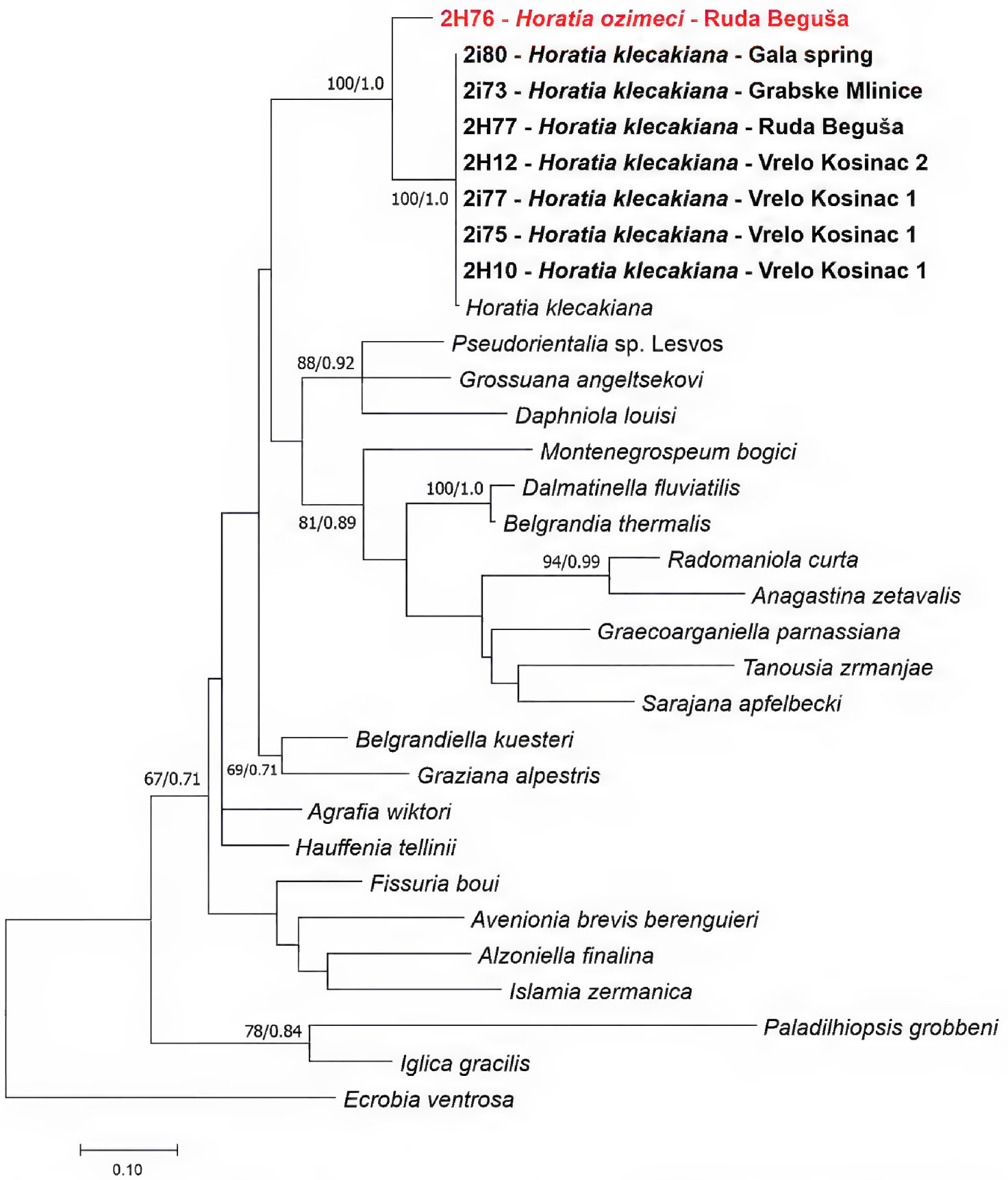

Figure 6. Maximum Likelihood tree inferred from mitochondrial COI. Bootstrap support above 60% and corresponding Bayesian probabilities are given. Bold indicates newly obtained sequences.

the whorls regularly tapering towards the aperture. Spire conically elevated, body whorl prominent. Smooth shell surface covered with blunt regular axial growth lines more prominent at the body whorl. Aperture almost rounded, tear-shaped, shortly attached to the body whorl by weak grove. Labral lateral profile backward protruded almost straight, columellar margin slightly sinuated at its middle. Umbilicus open.

Measurements of holotype of *H. stygorumina*: Table 2.

Soft parts morphology and anatomy. Not known.

Derivatio nominis. The specific epithet *stygorumina* is derived from the stygobiont habitat and from the type locality: cave Mali Rumin also referring to the name of the nearby settlement Rumin.

Distribution and habitat. Known only from the type locality, where empty shells can be found in the cave sediments, as well as in the sandy sediments of the intermittent spring. The shells are washed out from their stygobiont habitat. The cave is 50 m long, with two branches, acting as an intermittent overflow of the larger permanent spring Vrilo Rumin, situated 730 m east-southeast. Both springs are draining karstwater from middle part of Livansko Polje Basin (Bosnia and Herzegovina) Under Dinara Mts. towards upper Cetina River Valley. The following Hydrobiidae were detected in the locality: *Horatia klecakiana*, *Orientalina curta germari*, *Kerkia jadertina sinjana* (Kuščer, 1933) and a Montesieriid species of *Paladilhiopsis* Pavlović, 1913 and/or *Lanzaia* Brusina, 1906.

Discussion

All four valid species of *Horatia* described so far inhabit springs, but they have eyes and more or less pigmented soft parts, and can be classified as crenobiotic, at most as stygophiles (as defined by Culver and Pipan 2009, 2014). *H. ozimeci* sp. nov., with neither eyes nor any pigment, seems the first typically stygobiont *Horatia*. This single live specimen was most probably washed out from the cave together with few empty shells. Already Bourguignat (1887) noted the high variability of the shell of *H. klecakiana* (Fig. 1); later Radoman (1966, 1983) described and illustrated also the high variability of the penis in this species. The most characteristic feature of the shell of *H. ozimeci* is its partial scalarity – the open coil at the terminal part of the body whorl. There have been found also empty shells with the higher, conically elevated spire and more whorls visible within the umbilicus. Few empty shells found in the type locality were entirely scalariform, but they need not belong to *H. ozimeci*. Scalarity is characteristic for a few truncatelloidean species, e.g. *Gocea ochridiana* Hadžišče, 1956 from the Ochrid Lake (Radoman 1983), and several species from the Baikal Lake (Sitnikova et al. 2001; Clewing et al. 2015) but sometimes may be phenotypically determined (e.g. parasites, untypical chemistry: e.g. Fretter and Graham 1962), but is also typical feature of some species. In our case this morphological character is accompanied by molecular distinctness.

So far *H. klecakiana* was found only in the Cetina Valley and Livansko Polje Basin (Fig. 2). One of our sites was placed in the Ruda Valley and the second at Grab valley (both are left tributary of Cetina River); thus they are the first *H. klecakiana* localities outside the Cetina Valley and Livansko Polje.

High bootstrap support and p-distance between *H. ozimeci* and *H. klecakiana* confirm that they are two distinct species, belonging to the same genus *Horatia*. The evidence of species distinctness is especially strong since the two taxa occur in sympatry (inside the spring zone). The complete lack of polymorphism in the studied fragment

of COI in the specimens of *H. klecakiana* from its five sequenced populations additionally strengthens the molecular difference between *H. klecakiana* and *H. ozimeci* as delimiting distinct species.

The second stygobiotic species *H. stygorumina* sp. nov. is known only as empty shells from cave sediments of its type locality, 17 km from the locality of *H. ozimeci* sp. nov. Despite the similarities in the shell morphology of both species, suggesting their congeneric position, the second species differs from *H. ozimeci* sp. nov. by more elevated conical spire and more close-set last whorl. The stygobiotic *Horatia* forms can be found in most of the large springs at left tributary of upper Cetina River (springs: Rumin, Kosinac, Gala, Beguša, Grab). It may suggest possibly a similar evolutionary adaptation as we can see in the geographically close *H. ozimeci* sp. nov.

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